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INTERACTION OF THE CYCLIC-AMP-SPECIFIC PHOSPHODIESTERASE PDE4D5 WITH RACK1

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/135,035, filed May 20, 1999, which is hereby incorporated herein by reference.

BACKGROUND OF THE INVENTION

This invention relates to drug development. More particularly, the invention relates to type-specific modulators of cyclic AMP-specific phosphodiesterases.

Modulation of the levels of the second messenger adenosine cyclic 3',5'-monophosphate (cyclic AMP or cAMP) in cells is important in the regulation of numerous physiological processes, including those in the immune/inflammatory systems, vascular smooth muscle, and the brain. Cyclic nucleotide phosphodiesterases (PDEs) are a diverse family of enzymes that hydrolyze cAMP and guanosine cyclic 3',5'-monophosphate (cyclic GMP or cGMP) and thus play an important role in modulating cAMP levels. J.A. Beavo, 75 Physiol. Rev. 725-748 (1995). The cAMP-specific phosphodiesterases (PDE4s) can be differentiated from other PDEs by sequence homology of the catalytic region of the enzymes, M.D. Houslay et al., 44 Advances in Pharmacology 225-342 (1998), and by their ability to be specifically inhibited by the drug 4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidinone ("rolipram"). Rolipram and other specific PDE4 inhibitors have been shown to have anti-depressant, anti-inflammatory, and smooth-muscle relaxant activity in humans. M.D. Houslay et al., 44 Advances in Pharmacology 225-342 (1998). The PDE4 enzymes are also characterized by the presence of unique regions of amino acid sequence outside the catalytic region of the proteins, which are called upstream conserved regions 1 and 2 (UCR1 and UCR2) and are located in the amino-terminal half of the proteins. G. Bolger et al., 13 Mol. Cell Biol. 6558-6571 (1993). The PDE4s are comprised of a large family of isoforms, encoded by four different genes (*PDE4A, PDE4B,*

PDE4C, and *PDE4D*) in humans, with additional diversity being generated by alternative mRNA splicing. M.D. Houslay et al., 44 Advances in Pharmacology 225-342 (1998). All the catalytic regions of different isoforms that are encoded by the same gene are identical. In addition, the catalytic regions encoded by each of the four PDE4 genes are extremely similar. Therefore, drugs that act at the catalytic site of one of the PDE4 isoforms will inhibit all PDE4 isoforms. To develop isoform-specific inhibitors, compounds are identified that interact with unique region of each isoform, not with the catalytic region.

Five different isoforms encoded by the human *PDE4D* gene have been characterized, G. Bolger et al., 13 Mol. Cell Biol. 6558-6571 (1993); G. Nemoz et al., 384 FEBS Lett. 97-102 (1996); G.B. Bolger et al., 328 Biochem J. 539-548 (1997), all of which appear to be conserved among mammals. C. Sette et al., 269 J. Biol. Chem. 18271-18274 (1994); S.C. Jin et al., 273 J. Biol. Chem. 19672-19678 (1998). The five isoforms differ by the substitution of unique blocks of amino acids at the amino-terminal regions of their respective proteins. G.B. Bolger et al., 328 Biochem J. 539-548 (1997). The two smaller PDE4D isoforms, PDE4D1 and PDE4D2, are located exclusively in the cytosolic fraction of the cell. G.B. Bolger et al., 328 Biochem J. 539-548 (1997); S.C. Jin et al., 273 J. Biol. Chem. 19672-19678 (1998). The larger isoforms PDE4D3, PDE4D4, and PDE4D5 are each found both in the cytosol as well as in association with cellular particulate fractions. G.B. Bolger et al., 328 Biochem J. 539-548 (1997); S.C. Jin et al., 273 J. Biol. Chem. 19672-19678 (1998). The functional consequences of this diversity are poorly understood. However, the PDE4D3 isoform is a substrate for protein kinase A (PKA), which serves to activate this isoform. C. Sette et al., 269 J. Biol. Chem. 18271-18274 (1994); C. Sette et al., 269 J. Biol. Chem. 9245-9252 (1994); C. Sette & M. Conti, 271 J. Biol. Chem. 16526-16534 (1996); R. Hoffmann et al., 333 Biochem J. 139-149 (1998).

There has been intense interest in developing inhibitors of PDEs for use as therapies for human disease. The PDE

inhibitor, theophylline, has been on the market for many years for the treatment of asthma and other pulmonary diseases. Theophylline, however, inhibits members of all PDE families, and thus has a number of potential serious side effects. Recent drug development has focused on the development of type-specific inhibitors. The best-known type-specific inhibitor is the PDE5 inhibitor, sildenafil (VIAGRA, Pfizer Inc.), the well-known drug that is marketed for erectile dysfunction. Selective PDE4 inhibitors are being synthesized and evaluated by a large number of drug companies. PDE4 inhibitors, such as the drug rolipram, have smooth-muscle relaxant, anti-inflammatory, pro-apoptotic, and immunomodulatory properties, and are being tested as therapeutic agents for a variety of diseases, including asthma, rheumatoid arthritis, and other disorders. The PDE4 inhibitor, ARIFLOW (SmithKline Beecham), is currently in Phase III trials for asthma. All PDE4 inhibitors that have been tested to date, however, inhibit all the various PDE4 isoforms relatively equally. This is because all the PDE4 inhibitors that have been tested to date work as competitive (or partially competitive) inhibitors of the catalytic site of the enzyme. Therefore, it would be advantageous to develop inhibitors that work outside of the catalytic site.

In view of the foregoing, it will be appreciated that providing compositions and methods for modulating the activity of cAMP-specific phosphodiesterases and methods for screening type-specific inhibitors of cAMP-specific phosphodiesterases would be a significant advancement in the art.

BRIEF SUMMARY OF THE INVENTION

It is an object of the present invention to provide compositions that modulate the activity of cAMP-specific phosphodiesterases.

It is also an object of the invention to provide compositions that are type-specific modulators of cAMP-specific phosphodiesterases.

It is another object of the invention to provide methods for screening type-specific inhibitors of cAMP-specific

phosphodiesterases.

It is still another object of the invention to provide methods for developing drugs that modulate the activity of the cAMP-specific phosphodiesterase, PDE4D5.

These and other objects can be addressed by providing a method for screening candidate drugs for possessing activity for altering the activity of a PDE4D5 that interacts with RACK1 comprising detecting inhibition or stimulation by one or more of the candidate drugs of the interaction of the PDE4D5 with RACK1. In an illustrative embodiment of the invention, detecting inhibition or stimulation by one or more of the candidate drugs of the interaction of the PDE4D5 with RACK1 comprises

(a) forming a complex of a peptide with RACK1, wherein the peptide has a sequence that permits formation of a complex between the peptide and RACK1 that mimics the interaction of PDE4D5 with RACK1;

(b) contacting the complex with the one or more candidate drugs, and

(c) detecting inhibition or stimulation of the formation of the complex between the peptide and RACK1.

Preferably, the peptide is a member selected from the group consisting of SEQ ID NO:18, SEQ ID NO:34, SEQ ID NO:48, and mixtures thereof.

A method for altering the activity of a PDE4D5 that interacts with RACK1 comprises contacting RACK1 with a peptide that has a sequence that permits formation of a complex between the peptide and RACK1, wherein the complex mimics the interaction of PDE4D5 with RACK1, such that the complex is formed and formation of the complex inhibits the interaction between PDE4D5 and RACK1 or stimulates the activity of PDE4D5.

In another illustrative embodiment of the invention, a peptide for inhibiting interaction between PDE4D5 and RACK1, wherein the peptide is a member selected from the group consisting of SEQ ID NO:18, SEQ ID NO:34, SEQ ID NO:48, and mixtures thereof.

Further, a composition for pharmaceutical use of the peptide comprises an admixture of:

(a) a peptide for inhibiting interaction between PDE4D5 and RACK1, wherein the peptide is a member selected from the group consisting of SEQ ID NO:18, SEQ ID NO:34, SEQ ID NO:48, and mixtures thereof; and

(b) a pharmaceutically acceptable carrier.

In still another illustrative embodiment of the invention, a method for treating a condition that is susceptible of being ameliorated by a type-specific inhibitor of PDE4D5 comprises administering an effective amount of a composition comprising:

(a) a peptide for inhibiting interaction between PDE4D5 and RACK1, wherein the peptide is a member selected from the group consisting of SEQ ID NO:18, SEQ ID NO:34, SEQ ID NO:48, and mixtures thereof; and

(b) a pharmaceutically acceptable carrier.

Yet another illustrative embodiment of the invention comprises a method for obtaining a peptide that inhibits interaction of PDE4D5 and RACK1 comprising:

(a) determining a region of PDE4D5 that interacts with RACK1 in an interaction portion thereof; and

(b) synthesizing a peptide that interacts with the interaction portion of RACK1.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

FIG. 1 shows a schematic diagram of the amino-terminal region of PDE4D5. The line diagram at the top represents the five human PDE4D isoforms. G.B. Bolger et al., 328 Biochem J. 539-548 (1997). The numbers 1-5 represent isoforms PDE4D1 through PDE4D5, respectively. The heavy bar indicates sequences homologous to those in other PDE4 isoforms, with the strongest regions of conservation (the catalytic region and Upstream Conserved Regions 1 and 2 [UCR1 and UCR2]) indicated by the cross-hatched areas. The thin, branched lines adjacent to the numbers indicate sequence regions unique to each isoform. The thin lines merge where the sequences of the various isoforms join that of the shared sequence. The PDE4D2 isoform begins in the middle of UCR2, at a methionine that is internal to the other four isoforms. The bottom of the figure

shows the amino acid sequence (in single-letter code) of the unique 88 amino-terminal region of PDE4D5 (SEQ ID NO:1). The numbers immediately above the sequence indicate the amino acid co-ordinates. Amino acid residues subjected to site-directed mutagenesis (FIGS. 6C and 7B, below) are underlined (SEQ ID NO:2 through SEQ ID NO:11). The small arrows immediately above the sequence indicate amino acid residues that, when mutated, block the interaction with RACK1 (FIGS. 6C and 7B, below). The numbers below the sequence indicate the amino-terminal ends of the nested deletion constructs (N1, N2, etc.) used in FIGS. 6B and 7A (SEQ ID NO:12 through SEQ ID NO:15). WT indicates the full-length construct (SEQ ID NO:1). Regions of amino acid residues removed from deletion constructs D1 and D2 (SEQ ID NO:16 and SEQ ID NO:17) are also shown.

FIG. 2 shows a filter β-galactosidase assay demonstrating interaction of PDE4D5 with the receptor for activated protein C kinase (RACK1), but not with the related beta-prime coatomer protein (β'-COP). The PDE4D5 cDNA was cloned into pLEXAN to produce fusions with the LexA DNA-binding domain. Various WD-repeat proteins were cloned into pGADN to produce fusions with the GAL4 activation domain. *S. cerevisiae* cells containing the appropriate plasmids were patched onto plates that selected for both plasmids and subjected to a filter β-galactosidase assay, as described in G.B. Bolger, in Protein Targeting Protocols 101-131 (R.A. Clegg ed. 1998). Positive results in the assay produce a change in the color of the patches from pink to blue. The bottom two patches serve as internal positive and negative standards, respectively (the oncoproteins RAS^{v12} and RAF (A.B. Vojtek et al., 74 Cell 205-214 (1993)), and the vectors without inserts). As a positive control, the GAL4 activation domain fusion of β'-COP was shown to bind to LexA-α-COP in a two-hybrid test (data not shown). Blue patches were obtained with PDE4D5 + RACK1 and the positive control.

FIGS. 3A and 3B show immunoblots of extracts of untransfected COS7 cells probed with PDE4D-specific antibody (FIG. 3A) or RACK1-specific antibody (FIG. 3B). In FIG. 3A,

extracts from untransfected COS7 cells were fractionated (see Experimental Procedures) and then immunoblotted with the PDE4D antibody. G.B. Bolger et al., 328 Biochem J. 539-548 (1997). This identified in the high-speed supernatant (S2) fraction two immunoreactive species of 95±3 and 105±2 kDa. On the basis of co-migration with recombinant species, these represented PDE4D3 and PDE4D5, respectively. G.B. Bolger et al., 328 Biochem J. 539-548 (1997). PDE4D5 and, to a lesser extent, PDE4D3 were also found in the low-speed (P1) and high-speed (P2) pellet fractions. In FIG. 3B, extracts from untransfected COS7 cells were fractionated and then immunoblotted with the RACK1 antibody. This identified in the high-speed supernatant fraction a single immunoreactive species of 36±1 kDa consistent with RACK1. D. Ron et al., 91 Proc. Nat'l Acad. Sci. U.S.A. 839-843 (1994). A similar immunoreactive species was also found in both the low-speed (P1) and high-speed (P2) pellet fractions.

FIG. 3C shows an immunoblot of cytosolic fractions from untransfected COS7 cells subjected to immunoprecipitation with PDE4D-specific antibody (lanes marked "4D") or RACK1-specific antibody (lanes marked "RACK1"); the immunoprecipitates were then separated by SDS polyacrylamide gel electrophoresis, and the upper half of the gel was immunoblotted with the PDE4D-specific antibody and the lower half with the RACK1-specific antibody. Cytosolic (S2) fractions were prepared from untransfected COS7 cells and either immunoblotted directly (lanes marked "none"), or subjected to immunoprecipitation with either the PDE4D antibody (lanes marked "4D") or the RACK1 antibody (lanes marked "RACK1"). The fractions/immunoprecipitates were then separated by SDS-PAGE. The upper half of the gel was immunoblotted with the PDE4D-specific antibody. The arrows indicate the location of PDE4D3 and PDE4D5 as 95 and 105 kDa species, respectively. The lower half of the gel was immunoblotted with the RACK1-specific antibody, and the position of the 36 kDa RACK1 species is indicated. In each case, analyses were performed on cells treated (lanes marked "+") or not treated (lanes marked "-"), with the PKC-activating phorbol ester, phorbol-12-myristate-

13-acetate (PMA) (10 mM).

FIGS. 3D-F show immunoblots of the combined pellet (lanes marked "p") and S2 (lanes marked "s") fractions of untransfected COS7 cells treated with 10 mM phorbol-12-myristate-13-acetate (PMA); immunoblots were probed with protein kinase C (PKC) specific antibody (FIG. 3D), RACK1-specific antibody (FIG. 3E), or PDE4D-specific antibody (FIG. 3F). Cells were harvested at the indicated times (in minutes), fractionated as in FIGS. 3A-C and subjected to SDS-PAGE, followed by immunoblotting for PKCα (FIG. 3D), RACK1 (FIG. 3E) and PDE4D (FIG. 3F). All data are typical of experiments done at least three times. Each lane on the gels represents 50 µg of protein, with PDE activities in the range of 25-35 pmol/min/mg protein. Equal amounts of protein from the P1, P2, and S2 fractions were analyzed. The PDE4D antibodies (either polyclonal or monoclonal) did not immunoprecipitate purified GST-RACK1. The RACK1 antibody did not immunoprecipitate purified MBP-PDE4D5 (data not shown).

FIGS. 4A and 4B show immunoblots of cytosolic extracts of Jurkat (FIG. 4A), HEK-293, 3T3-F442A, and SK-N-SH cell lines (FIG. 4B) either immunoblotted directly (lanes marked "T") or subjected to immunoprecipitation with the RACK1-specific antibody (lanes marked "rIP" or "i") or a non-specific mouse antiserum (lanes marked "n"); the upper half of the gel was immunoblotted with PDE4D-specific antibody, and the lower half of the gel was immunoblotted with RACK1-specific antibody. Cytosolic extracts were prepared from the Jurkat, HEK-293, 3T3-F442A and SK-N-SH cell lines. They were either immunoblotted directly (lanes marked "T"), or subjected to immunoprecipitation with the RACK1 antibody (lanes marked "rIP" or "i") or a non-specific mouse antiserum (lanes marked "n"). The fractions/immunoprecipitates were then separated by SDS-PAGE. The upper half of the gel was immunoblotted with the PDE4D-specific antibody. The arrows indicate the location of PDE4D3 and PDE4D5 as 95 and 105 kDa species, respectively. The lower half of the gel was immunoblotted with the RACK1-specific antibody, and the position of the 36 kDa RACK1 species is indicated. Each lane on the gels represents 50 µg

of protein, with PDE activities in the range of 25-35 pmol/min/mg protein. RACK1 was also co-immunoprecipitated with PDE4D5 when the initial immunoprecipitation was performed with the PDE4D5-specific antibody, as demonstrated by immunoblotting of PDE4D5 immunoprecipitates with the RACK1 antibody (data not shown).

FIGS. 5A and 5B show SDS polyacrylamide gel electrophoresis separations of glutathione-S-transferase (GST) and a fusion between GST and RACK1 (GST-RACK1) (FIG. 5A); and fusion between maltose-binding protein (MBP) and PDE4D3 (MBP-4D3) or PDE4D5 (MBP-4D5) (FIG. 5B). Fusions between GST and RACK1, and also between MBP and PDE4D3 or PDE4D5, were expressed and purified from *E. coli* (see Experimental Procedures). GST alone (i.e., not as a fusion) was expressed and purified in an identical manner. Cell lysates (lanes marked "b") and purified proteins (lanes marked "e") obtained after elution from the appropriate affinity column were separated by SDS-PAGE and stained with Coomassie blue. The species were purified to apparent homogeneity as analyzed by SDS-PAGE. The positions of the arrows marks the relative molecular weight of the purified proteins, as follows: GST: M_r 27.3±1.1 kDa, GST-RACK1: 59.8±1.4 kDa, MBP-4D3: 128±3.8 kDa, MBP-4D5: 135.3±2.6 kDa. These data are typical of experiments done at least three times.

FIG. 5C shows the interaction of *E. coli*-purified recombinant PDE4D5 and RACK1 tested in an enzyme linked immunosorbent assay (ELISA). The MBP-PDE4D5 fusion bound to GST-RACK1 in a dose-dependent manner, with an EC_{50} of 7.4±1.1 pM (mean±SD; n=3 separate experiments). As a control, parallel experiments were performed for MBP-PDE4D3.

FIG. 5D shows dose-response curves calculated for the inhibition of PDE4D5 by rolipram at a concentration of substrate (cAMP) of 1.0 μM. Assays were performed on MBP-PDE4D5 ("4D5-MBP") alone, and also on MBP-PDE4D5 complexed with GST-RACK1. Assays were performed using an excess of GST-RACK1 so that all of the PDE4D5 would be complexed with RACK1 (see Experimental Procedures). In pull-down experiments, all of the PDE4D5 could be shown to complex with GST-RACK1 under

these conditions (data not shown). As a control, assays were performed with GST alone, added at comparable levels. The IC_{50} values for rolipram inhibition were 0.13±0.1, 0.16±0.05, and 0.52±0.07 μM for MBP-PDE4D5 alone, MBP-PDE4D5 mixed with GST, and MBP-PDE4D5 mixed with GST-RACK1, respectively (mean±SD, n=3). These values are significantly different (MBP-PDE4D5 alone compared to MBP-PDE4D5 complexed with GST-RACK1; $P<0.005$, T-test). Protein assays were performed and molar concentrations were determined on the basis of the calculated molecular weights.

FIGS. 6A-C show pull-down assays of recombinant forms of PDE4D5, i.e. wild-type recombinant PDE4D5 isoforms (FIG. 6A) and deletion mutants of PDE4D5 (FIGS. 6B and 6C), with RACK1. In these experiments, various PDE4D isoforms (or mutant forms thereof) were expressed in COS7 cells. Cytosolic extracts were prepared from the cells, mixed with GST-RACK1, and then subjected to affinity absorption on glutathione agarose beads, followed by SDS-PAGE and immunoblotting. FIG. 6A shows a pull-down experiment performed with wild-type recombinant PDE4D isoforms, wherein PDE4D3 or PDE4D5 was expressed in COS7 cells, and then subjected to "pull-downs" with GST-RACK1 (lanes marked "rg") or GST alone (lanes marked "g"). After affinity absorption, the material on the beads was immunoblotted with the PDE4D antibody. Cytosolic extracts from the cells (i.e., not subjected to "pull-downs") were run as standards (lanes marked "ly"). In cells transfected with vector alone ("mock"), GST-RACK1 pulled down a single 105 kDa species consistent with PDE4D5. In cells transfected with a construct expressing PDE4D3, a 105 kDa band was seen, whose intensity was not different from that seen in the mock-transfected cells. In cells transfected with a construct expressing PDE4D5, there was a dramatic increase in the intensity of the PDE4D immunoreactive species pulled down by GST-RACK1. Expression of the PDE4D isoforms in the COS7 cells was measured by quantitative immunoblotting with the PDE4D monoclonal antibody, as described in G.B. Bolger et al., 328 Biochem J. 539-548 (1997). The expression of the various PDE4D constructs was similar and identical amounts were used

in comparative experiments. FIG. 6B shows a pull-down experiment performed with constructs encoding deletions in PDE4D5. The regions of PDE4D5 included in the various constructs are shown in FIG. 1. All the constructs encoded a VSV epitope at the carboxy-terminal end of the protein, G.B. Bolger et al., 328 Biochem J. 539-548 (1997); R. Hoffmann et al., 333 Biochem J 139-149 (1998), which could be detected by an anti-VSV antibody, T.E. Kreis, 5 EMBO J. 931-941 (1986), using methods described by in G.B. Bolger et al., 328 Biochem J. 539-548 (1997); R. Hoffmann et al., 333 Biochem J 139-149 (1998). The expression of the construct was monitored by immunoblotting of cytosolic extracts with the anti-VSV antibody (FIG. 6B, upper panel; "lysate"). This allowed detection of the transfected species without background from endogenous PDE4D5. Pull-down experiments were performed with GST-RACK1, followed by immunoblotting of the material on the beads with the VSV-antibody (FIG. 6B, lower panel; "Rack1-bound"). Lane "m" represents mock transfections (vector only). FIG. 6C shows co-immunoprecipitations of RACK1 and PDE4D5 mutants. Constructs encoding mutants of PDE4D5 with Asn22 mutated to Ala (SEQ ID NO:6; lanes marked "22"), or with Trp24 mutated to Ala (SEQ ID NO:8; lanes marked "24") were expressed in COS7 cells. Wild-type (unmutated) PDE4D5 was expressed in parallel transfections (lanes marked "wt"). Cytosolic fractions were immunoblotted directly (lanes marked "lysate"), or were immunoprecipitated with the RACK1 antibody (lanes marked "RACK ippt"), or with a non-specific mouse antiserum (lanes marked "ns-Ab ippt"). The samples were then immunoblotted with the PDE4D antibody. The migration of PDE4D5 on the gel is indicated. All data are typical of experiments done at least three times.

FIGS. 7A and 7B show yeast two-hybrid analysis of PDE4D5 deletions and point mutations. In FIG. 7A, plasmids encoding fusions between the DNA-binding domain of LexA and various amino-terminal deletions of PDE4D5 were tested for their ability to interact with RACK1, expressed as a fusion with the GAL4 activation domain (right column, "pGADN-RACK1"). The identical LexA fusions were tested for their ability to

interact with the GAL4 activation domain alone (left column, "pGADN"). The regions of PDE4D5 included in the various constructs are shown in FIG. 1. Also shown is the interaction generated by a LexA fusion containing the unique 88 amino acid amino-terminal region of PDE4D5 ("NT"). The interactions were tested with the filter β-galactosidase assay used in FIG. 2. In FIG. 7B, individual amino acid residues in the amino-terminal region of PDE4D5 were mutated to alanine, and the resulting constructs were expressed as LexA fusions and tested for their ability to interact with pGADN-RACK1. Also included as controls are LexA fusions of unmutated PDE4D5 ("wt"), and the signal produced with pGADN-RACK1 and LexA alone ("nb").

DETAILED DESCRIPTION

Before the present compositions and methods for type-specific modulation of cAMP-specific phosphodiesterases and methods for screening type-specific inhibitors of cAMP-specific phosphodiesterases are disclosed and described, it is to be understood that this invention is not limited to the particular configurations, process steps, and materials disclosed herein as such configurations, process steps, and materials may vary somewhat. It is also to be understood that the terminology employed herein is used for the purpose of describing particular embodiments only and is not intended to be limiting since the scope of the present invention will be limited only by the appended claims and equivalents thereof.

The publications and other reference materials referred to herein to describe the background of the invention and to provide additional detail regarding its practice are hereby incorporated by reference. The references discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

It must be noted that, as used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise.

In describing and claiming the present invention, the following terminology will be used in accordance with the definitions set out below.

As used herein, "comprising," "including," "containing," "characterized by," and grammatical equivalents thereof are inclusive or open-ended terms that do not exclude additional, unrecited elements or method steps. "Comprising" is to be interpreted as including the more restrictive terms "consisting of" and "consisting essentially of."

As used herein, "consisting of" and grammatical equivalents thereof exclude any element, step, or ingredient not specified in the claim.

As used herein, "consisting essentially of" and grammatical equivalents thereof limit the scope of a claim to the specified materials or steps and those that do not materially affect the basic and novel characteristic or characteristics of the claimed invention.

As used herein, "ORF" means open reading frame.

As used herein, "WD-repeat" means a protein sequence motif containing repetitive sequences bound by tryptophan (signified by "W" is standard single-letter code) and aspartic acid (signified by "D" is standard single-letter code) residues.

As used herein, "SD" means standard deviation.

As used herein, "*S. cerevisiae*" means *Saccharomyces cerevisiae*.

As used herein, "single-letter code" and similar terms refer to single-letter designations for the 20 amino acid residues found in peptides and proteins, as follows: A - alanine, C - cysteine, D - aspartic acid, E - glutamic acid, F - phenylalanine, G - glycine, H - histidine, I - isoleucine, K - lysine, L - leucine, M - methionine, N - asparagine, P - proline, Q - glutamine, R - arginine, S - serine, T - threonine, V - valine, W - tryptophan, and Y - tyrosine.

The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as dizziness and the like, when

administered to a human. Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans.

The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or aqueous solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions.

As used herein, "effective amount" means an amount of a drug or pharmacologically active agent that is nontoxic but sufficient to provide the desired local or systemic effect and performance at a reasonable benefit/risk ratio attending any medical treatment. An effective amount of a peptide for inhibiting the interaction between PDE4D5 and RACK1 is an amount sufficient to inhibit the interaction to a selected level.

As used herein, "administering" and similar terms mean delivering the composition to the individual being treated such that the composition is capable of being circulated systemically to the parts of the body where the peptide can inhibit the interaction between PDE4D5 and RACK1. Thus, the composition is preferably administered to the individual by systemic administration, typically by subcutaneous, intramuscular, or intravenous administration, or intraperitoneal administration. Injectables for such use can be prepared in conventional forms, either as a liquid solution or suspension or in a solid form suitable for preparation as a solution or suspension in a liquid prior to injection, or as an emulsion. Suitable excipients include, for example, water, saline, dextrose, glycerol, ethanol, and the like; and if desired, minor amounts of auxiliary substances such as wetting or emulsifying agents, buffers, and the like can be added.

Regulation of the levels of the second messenger cAMP has the potential to influence multiple processes in cells and tissues. PDE4 enzymes have been implicated in numerous cellular functions in the brain, airway smooth muscle, endocrine tissues, and in the immune/inflammatory systems (for review see M.D. Houslay et al., 44 Advances in Pharmacology 225-342 (1998)). However, the regulation of many PDE4 isoforms is poorly understood. A novel PDE4 isoform, PDE4D5, which is expressed in numerous cell lines and also in the brain, G.B. Bolger et al., 328 Biochem J. 539-548 (1997), has recently been isolated. It is demonstrated herein that PDE4D5 interacts specifically and at high affinity with the RACK1 WD-repeat "scaffold" protein. The interaction between these two proteins is demonstrated by multiple independent methods, including two-hybrid screening, pull-down assays with recombinant RACK1, and binding studies with purified recombinant proteins. It is also demonstrated by co-immunoprecipitation studies that native, endogenously expressed RACK1 and PDE4D5 interact in cells.

There is described herein the property of one PDE4 isoform, namely its ability to interact with the scaffold protein RACK1. No other PDE4 isoform interacts with RACK1. RACK1 interacts with the extreme amino-terminal region of PDE4D5, which is well outside its catalytic region. It is desirable to develop drugs that block this interaction, which in turn would modulate various functions of PDE4D5 in cells. These drugs would potentially be important in treating any disease for which type-selective PDE4 inhibitors are now being tested, but with potentially fewer side effects than current PDE4 inhibitors.

As used herein, "RACK1" means Receptor for Activated Protein C Kinase. The first identified functional role for RACK1 was as a protein capable of binding to various PKC isoforms after they had been activated through treatment of cells with either diacylglycerol or phorbol esters. D. Ron et al., 91 Proc. Nat'l Acad. Sci. U.S.A. 839-843 (1994); D. Ron & D. Mochly Rosen, 92 Proc. Nat'l Acad. Sci. U.S.A. 492-496 (1995); D. Ron et al., 270 J. Biol. Chem. 24180-24187 (1995);

D. Mochly Rosen, 268 Science 247-251 (1995). It is a member of the large family of WD-repeat proteins, E.J. Neer et al., 371 Nature 297-300 (1994), which have numerous functions in cells. It is most homologous to the $G_s\beta$ subunit, which activates certain isoforms of adenylyl cyclase and various ion channels, E.J. Neer et al., 371 Nature 297-300 (1994); J.A. Pitcher et al., 257 Science 1264-1267 (1992), and which also serves as an "anchor" for the β-adrenergic receptor kinase (BARK; D.G. Lambright et al., 379 Nature 311-319 (1996)). X-ray diffraction studies of the $G_s\beta$ subunit have demonstrated that it is has a "propeller" structure, M.A. Wall et al., 83 Cell 1047-1058 (1995); I. Garcia Higuera et al., 35 Biochemistry 13985-13994 (1996), and the "blades" of the "propeller" are believed to be sites of interaction between $G_s\beta$ and other proteins. It is likely that RACK1 has a similar structure. D. Mochly Rosen et al., 88 Proc. Nat'l Acad. Sci. USA 3997-4000 (1991). The coatomer protein, β'-COP, is also a WD-repeat protein that also interacts with PKC isoforms. M. Csukai et al., 272 J. Biol. Chem. 29200-29206 (1997). It is shown herein that PDE4D5 does not bind to either $G_s\beta$ or β'-COP, demonstrating that PDE4D5 interacts specifically with RACK1 and not with WD-repeat proteins in general.

More recently, RACK1 has been shown to interact with the integrin β subunit, J. Liliental & D.D. Chang, 273 J. Biol. Chem. 2379-2383 (1998), and the protein tyrosyl kinase Src, ; B.Y. Chang et al., 18 Mol. Cell. Biol. 3245-3256 (1998). RACK1 may serve as a "scaffold" or "adaptor" protein for either Src or the integrin β subunit. Overexpression of RACK1 inhibits the tyrosine kinase activity of Src and inhibits the growth of NIH-3T3 cells. B.Y. Chang et al., 18 Mol. Cell. Biol. 3245-3256 (1998). The physiologic role of RACK1 in integrin function remains to be determined (see below).

Although RACK1 has been shown to interact with at least four different proteins, the mechanisms for these interactions appear to be quite different. PKC isoforms need to be activated by treatment with phorbol esters and Ca^{2+} before they can interact with RACK1. G. Scotland & M.D. Houslay, 308

Biochem. J. 673-681 (1995). This treatment is believed to produce a conformational change in the PKC enzyme, which exposes its C2 region, allowing it to interact with RACK1. D. Ron & D. Mochly Rosen, 92 Proc. Nat'l Acad. Sci. U.S.A. 492-496 (1995); D. Ron et al., 270 J. Biol. Chem. 24180-24187 (1995); D. Mochly Rosen, 268 Science 247-251 (1995). A number of domains within the C2 region of PKC are involved in its binding to RACK1. D. Ron & D. Mochly Rosen, 92 Proc. Nat'l Acad. Sci. U.S.A. 492-496 (1995); D. Ron et al., 270 J. Biol. Chem. 24180-24187 (1995). These domains show no obvious sequence homology to the region in the amino-terminus of PDE4D5 that are shown herein to be essential for its interaction with RACK1 (FIGS. 6A-C and 7A-B). The interaction of RACK1 with the β subunit of integrins also requires the stimulation of cells with PMA. J. Liliental & D.D. Chang, 273 J. Biol. Chem.. 2379-2383 (1998). This suggests that PKC activation is necessary for the interaction of the integrin β subunit with RACK1, or that PMA can directly promote the interaction. This interaction may involve a conformational change in RACK1, as the interaction between RACK1 and the integrin β subunit can only be demonstrated *in vitro* if a truncated form (WD-repeats 5 through 7, inclusive) of RACK1 is used.

In some ways, the interaction observed between PDE4D5 and RACK1 appears to resemble that between RACK1 and the SH2 region of Src. B.Y. Chang et al., 18 Mol. Cell. Biol. 3245-3256 (1998). Src interacts with full-length GST-RACK1 in pull-down assays, which is also true for PDE4D5 (FIGS. 3, 4, and 7). Co-immunoprecipitation of Src and RACK1 from cell lysates did not require PMA, which is also true for the interaction between RACK1 and PDE4D5. Therefore, RACK1 may interact with different proteins through different mechanisms, some of which involve phorbol-ester-induced conformational changes, and some of which apparently do not.

Several other PDE4 isoforms have also been demonstrated to bind to other signaling proteins, and it is of interest that these interactions also appear to be mediated by

sequences in the unique amino-terminal ends of the PDE4 proteins. The PDE4A4/5 isoform has been demonstrated to bind to proteins containing SH3 domains. J.C. O'Connell et al., 318 Biochem. J. 255-262 (1996). The PDE4A1 isoform is targeted to membranes, and the membrane targeting of this isoform is mediated by specific amino acids in its amino-terminal region. I. McPhee et al., 310 Biochem. J. 965-974 (1995); Y. Shakur et al., 292 Biochem. J. 677-686 (1993); Y. Shakur et al., 306 Biochem. J. 801-809 (1995);C.S. Rubin, 1224 Biochim. Biophys. Acta 467-479 (1994).

The physiologic implications of the RACK1:PDE4D5 interaction may be related to the ability of RACK1 to serve as a "scaffold" or "adaptor" protein that mediates the recruitment of PDE4D5 into a protein complex. A single "scaffold" or "adaptor" protein may interact with multiple different proteins, all of which can potentially be recruited into the complex. For example, AKAPs (A-kinase anchoring proteins) can interact with protein kinase A, PKC, and protein phosphatase 1. T. Pawson & J.D. Scott, 278 Science 2075-2080 (1997); D. Faulstich et al., 135 J. Cell Biol. 53-61 (1996). "Scaffold," "anchor," and "adaptor" proteins physically connect various signal transduction components, such as receptors, kinases, and elements of the cytoskeleton, into stable complexes. These complexes bring enzymes closer to their regulatory components or substrates, or closer to other components of a signaling network. T. Pawson & J.D. Scott, 278 Science 2075-2080 (1997). Therefore, the multidomain protein RACK1 may serve as a "scaffold" able to recruit a variety of signal transduction proteins. Some of RACK1's partners, such as PKC isoforms and integrins, may be recruited to the complex only upon activation by phorbol esters, while others, such as PDE4D5 and Src, may be constitutively associated with RACK1 in those cell types where both proteins are expressed. The functional significance of the association of PDE4D5 with RACK1 remains to be elucidated. The recruitment of PDE4D5 to a signaling complex may provide a potential mechanism for the modulation of cAMP levels in the

vicinity of the complex. This could turn regulate the activity of protein kinase A, which could influence the activity or function(s) of the complex, or of other adjacent cellular components.

EXPERIMENTAL PROCEDURES

Materials - A HeLa cell (Human HeLa S3 cells; American Type Culture Collection) two-hybrid library cloned into the *Eco*RI and *Xho*I sites of the pGADGH vector, G.J. Hannon et al., 7 Genes Dev. 2378-2391 (1993), was obtained from David Beach (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). This vector expresses proteins as fusions with the activation domain of the *S. cerevisiae GAL4* protein. Antibodies to RACK1 were obtained from Transduction Laboratories (Lexington, Kentucky). The RACK1 antibody detects a 36 kDa species in Jurkat cells. J. Liliental & D.D. Chang, 273 J. Biol. Chem.. 2379-2383 (1998); B.Y. Chang et al., 18 Mol. Cell. Biol. 3245-3256 (1998). A monoclonal antibody to human PDE4D proteins, which does not cross-react with other PDE4 species, and which has been described previously, G.B. Bolger et al., 328 Biochem J. 539-548 (1997), was obtained from Sharon Wolda, ICOS Corp. (Bothell, Washington). This antibody interacts with PDE4D3 and PDE4D5 with very similar affinity. G.B. Bolger et al., 328 Biochem J. 539-548 (1997). The PDE4D antibody also detects PDE4D species in rats and mice. G.B. Bolger et al., 328 Biochem J. 539-548 (1997). cDNA clones for $G_s\beta$ and beta-prime coatomer protein (β'-COP) were obtained from A.G. Gilman and K.J. Harrison-Lavoie, respectively. The L40 *S. cerevisiae* strain and the pBTM116 plasmid were obtained from A. B. Vojtek.

Two-hybrid screens - These experiments were performed using methods described in G.B. Bolger, in Protein Targeting Protocols 101-131 (R.A. Clegg ed. 1998). In brief, the full open reading frame (ORF) of the pPDE79 cDNA (GenBank accession number AF012073) encoding human PDE4D5, G.B. Bolger et al., 328 Biochem J. 539-548 (1997), was cloned into the *Not*I site of pLEXAN to generate pLEXAN79, which encodes a fusion between

PDE4D5 and the DNA-binding domain of the *E. coli* LexA protein. These constructs were prepared by the addition of *Not*I sites to the cDNAs by the use of PCR, as described in G. Bolger et al., 13 Mol. Cell. Biol. 6558-6571 (1993). pLEXAN is a derivative of pBTM116, P.L. Bartel & S. Fields, 254 Methods Enzymol. 241-263 (1995), with a *Not*I site inserted into the polylinker. Screens were performed with the HeLa two-hybrid cDNA library in the *S. cerevisiae* strain L40. A.B. Vojtek et al., 74 Cell 205-214 (1993). To screen the HeLa cDNA library, positive clones were initially selected for growth in the absence of histidine (without 3-amino-triazole), and then transferred to patches and assayed for *LacZ* activity using a filter β-galactosidase assay, as described in G.B. Bolger, in Protein Targeting Protocols 101-131 (R.A. Clegg ed. 1998). Library plasmid DNA was then isolated from the positives and then re-assayed for interaction with PDE4D5, using methods described in G.B. Bolger, in Protein Targeting Protocols 101-131 (R.A. Clegg ed. 1998).

For additional two-hybrid experiments, the full ORFs of all 5 human PDE4D isoforms, G.B. Bolger et al., 328 Biochem J. 539-548 (1997), and other cDNAs were cloned into the *Not*I site of pLEXAN, in a manner analogous to that described above for PDE4D5. Similarly, the full ORF of human RACK1 (F. Guillemot et al., 86 Proc. Nat'l Acad. Sci. U.S.A. 4594-4598 (1989) (GenBank accession number M24194)), obtained from a positive isolated in the two-hybrid screen, was cloned into the *Not*I site of pGADN, to produce pGADNRACK1. pGADN is a derivative of pGADGH, but with a *Not*I site inserted into the polylinker.

Generation of bacterial expression constructs - The full ORFs of PDE4D5, PDE4D3 and RACK1 were cloned into the *Not*I site of pMALN, to generate pMALPDE4D5 (also called pMALP79) and pMALPDE4D3 (also called pMALN43), respectively. pMALN is a derivative of pMALc2 (New England Biolabs, Beverly, Massachusetts; C. Guan et al., 67 21-30 (1988)), with a *Not*I site inserted into the polylinker. The full ORF of RACK1 was cloned into the *Not*I site of pGEX-5X-3 (Pharmacia, Piscataway, New Jersey; D.B. Smith & K.S. Johnson, 67 Gene 31-40 (1988))

to generate pGEXRACK1. All constructs named "pMAL . . ." generate fusions between the maltose-binding protein (MBP) and the amino-terminus of the protein encoded by the insert. Constructs named "pGEX . . ." constructs generate fusions between glutathione-S-transferase (GST) and the amino-terminus of the protein encoded by the insert.

Generation of COS7 cell expression constructs - The full ORF of PDE4D5 was cloned into the *Not*I site of pcDNA3 (Invitrogen, San Diego, California), to create pcDNAPDE4D5VSV (also called pcDNAN79VSV). In this construct, the insert is placed under the control of the cytomegalovirus intermediate early gene promoter. The full ORF of PDE4D3 was cloned into pCIneo to produce pCIneo-PDE4D3. G.B. Bolger et al., 328 Biochem J. 539-548 (1997). In both cases, a sequence corresponding to the vesicular stomatitis virus (VSV) glycoprotein epitope, T.E. Kreis, 5 EMBO J. 931-941 (1986), was added immediately downstream from the last codon of the PDE, to encode a carboxy-terminal fusion. The native PDE4D stop codon was removed in this process, but a synthetic stop codon was placed immediately downstream from the epitope sequence. The full ORFs of RACK1, PDE4D1, PDE4D2, PDE4D4, PDE4B1, PDE4B2, PDE4B3, and PDE4C2 were cloned into the *Not*I site of pcDNA3 (without a carboxyl-terminal epitope).

Generation of cDNAs encoding mutant forms of PDE4D5- To generate deletions in the amino-terminal region of PDE4D5, PCR was used to amplify various regions of PDE4D5, which were then cloned into pLEXAN or pcDNA3. *Not*I sites were added to the PCR primers to aid in cloning. To generate point mutations in PDE4D5, the full-length PDE4D5 cDNA was subjected to site-directed mutagenesis with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, California).

Verification of two-hybrid, expression and mutagenesis constructs- All PCR-generated or mutant constructs were verified by sequencing prior to use.

Growth of cell lines- All cell lines used in these experiments were obtained from the American Type Culture Collection (Manassas, Virginia). The lines were grown in

Dulbecco's modified Eagle's medium, supplemented with fetal calf serum and antibiotics.

Co-immunoprecipitations- COS7, SK-N-SH, Jurkat, 3T3-F442A or HEK 293 cells were harvested in 0.5 ml lysis buffer (55 mM Tris-HCl, pH 7.4, 132 mM NaCl, 22 mM sodium fluoride, 11 mM sodium pyrophosphate, 1.1 mM EDTA, 5.5 mM EGTA) containing complete protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, Indiana) and lysed with 8 strokes of a 26½ gauge needle attached to a disposable syringe. This method was used to permit processing of a large number of samples quickly. It produced complete lysis of cells on the basis of vital dye staining and absence of latent LDH activity. After lysis, cell debris was removed by centrifugation at 12,000 x *g* for 10 min.

For immunoprecipitation of RACK1, 500 µg of cleared cytosol was mixed with 30 µl of pre-equilibrated anti-mouse IgM agarose (Sigma Chemical Co., St. Louis, Missouri) and incubated for 30 min at 4° C. The beads were removed by centrifugation at 2,000 x *g* for 5 minutes and the cleared lysates incubated with 16 µl RACK1-specific antibody (Transduction Laboratories, Lexington, Kentucky) in the presence of anti-mouse IgM agarose beads for 3 hours at 4° C. For immunoprecipitation of PDE4D5 from COS7 cells, 500 µg of cleared cytosol was mixed with 30 µl of pre-equilibrated protein A agarose beads (Sigma) and incubated for 30 min at 4° C. The beads were removed and lysates were incubated for 3 hours at 4°C with 16 µl of monoclonal anti-PDE4D antibody, G.B. Bolger et al., 328 Biochem J. 539-548 (1997), in the presence of protein A agarose beads. In both cases, the beads were then collected by centrifugation (2,000 x *g* for 5 minutes) and washed three times with lysis buffer. Co-immunoprecipitation of PDE4D with RACK1 was analyzed by immunoblotting with the PDE4D-specific monoclonal antibody and the RACK1-specific antibody.

Expression of glutathione S-transferase (GST) and maltose-binding protein (MBP) fusions in E. coli- Cultures of *E. coli* JM109 containing pGEXRACK1, pMALPDE4D3 or pMALPDE4D5

were induced with 1mM isopropyl-β-D-thiogalactopyranoside (IPTG; Boehringer Mannheim, Indianapolis, Indiana) for 4 hours at 30°C. Bacteria were harvested by centrifugation at 2,500 x *g* for 10 minutes at 4°C, and the bacterial pellet was frozen at -80°C overnight. The bacterial pellets were resuspended in 10 ml of ice-cold resuspension buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, and complete protease inhibitor cocktail) and sonicated with 4 x 30 sec bursts at maximal setting. Triton X-100 was added to a final concentration of 0.02% and cell debris was then removed by centrifugation at 15,000 x g for 10 minutes at 4°C. The cleared supernatant was incubated with 0.1 volume of pre-equilibrated glutathione sepharose beads (for GST fusions) or amylose resin (for MBP fusions) on an orbital shaker for 30 minutes at 4°C. The beads were collected by centrifugation at 2,000 x *g* for 1 minute and washed three times with ice-cold resuspension buffer. The fusion proteins were eluted by the addition of 5 mM glutathione, 50 mM Tris-HCl, pH 8.0 (for GST fusions) or 10 mM maltose, 50 mM Tris-HCl, pH 8.0 (for MBP fusions) and dialyzed three times against 50 mM Tris-HCl pH 8.0, 100 mM NaCl, and 5% glycerol. The purified fusion proteins were stored at -80°C until required.

RACK1 Pulldown Assays- COS7 cells were transfected with either 10 µg of plasmid pcDNAPDE4D5VSV or 10 µg of plasmid pCIneo-PDE4D3. Transfections were done as described in G.B. Bolger et al., 328 Biochem J. 539-548 (1997); I. McPhee et al., 310 Biochem. J. 965-974 (1995). After 72 hours, cells were harvested and lysed with 8 strokes of a 26½ gauge needle in 0.5 ml lysis buffer. Cell debris was removed by centrifugation (12,000 x *g* for 10 minutes at 4°C) and 500 µg of cleared lysate was incubated with GST or GST-RACK1 and 60 µl of glutathione-Sepharose beads for 1 hour at 4°C. Beads were pelleted by centrifugation at 2,000 x *g* for 5 minutes at 4°C and washed three times in lysis buffer. Protein complexes were eluted by the addition of 5 mM glutathione, 50 mM Tris-HCl, pH 8.0 and co-precipitation of PDE4D5 was analyzed by immunoblotting with an anti-VSV monoclonal antibody. T.E.

Kreis, 5 EMBO J. 931-941 (1986). In two cases (see below) immunoblotting was performed with the PDE4D monoclonal antibody.

ELISA Protein Interaction Assay- Reacti-Bind glutathione coated ELISA plates (Pierce, Rockford, Illinois) were treated with 1 mg of purified GST or GST-RACK1 for 16 hours at 4°C and then washed three times with 100 µl/well of wash buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20). Dilutions of the MBP fusions of PDE4D3 and PDE4D5 were incubated with immobilized GST or GST-RACK1 for 3 hours at room temperature and the wells were then washed three times with 100 µl/well of ice-cold wash buffer. Protein complexes were fixed with the addition of 100 µl/well 4% (v/v) paraformaldehyde in PBS for 30 minutes at 4°C. Paraformaldehyde fixation was used at this step because of concern that the complexes might dissociate during the subsequent detection procedure. However, it was determined subsequently that the binding of PDE4D5 and RACK1 was so avid that the addition of paraformaldehyde made little difference. After fixation, protein complex formation was detected by the addition of anti-PDE4D monoclonal antibody (1:10,000 (v/v) in dilution buffer [10 mM Tris-HCl, pH 7.4, 150 mM NaCl]) for 1 hour at room temperature, followed by alkaline phosphatase conjugated anti-mouse IgG (Sigma Chemical Co., St. Louis, Illinois; 1:2000 (v/v) in dilution buffer) for a further hour at room temperature. Immunoreactivity was visualized with the BCIP Microwell 2 Component Phosphatase Substrate System (KPL, Inc., Gaithersburg, Maryland) following the manufacturer's instructions and quantified using a MRX microplate reader (Dynex Technologies, Inc., Chantilly, Virginia) set at a test wavelength of 630 nm. RACK1 binding was stated as being the amount of PDE4D immunoreactivity found associated with GST-RACK1 minus that associated with GST alone and was expressed as arbitrary units.

Preparation and fractionation of tissue homogenates- These preparations were made as described in G.B. Bolger et al., 328 Biochem J. 539-548 (1997); I. McPhee et al., 310 Biochem. J. 965-974 (1995); Y. Shakur et al., 292 Biochem. J. 677-686 (1993); Y. Shakur et al., 306 Biochem. J. 801-809

(1995); G.B. Bolger et al., 271 J. Biol. Chem. 1065-1071 (1996); E. Huston et al., 271 J. Biol. Chem. 31334-31344 (1996). Confluent cultures of COS7 cells were scraped into 0.8 ml of lysis buffer and lysed with 20 strokes of a Dounce homogenizer equipped with a tight-fitting pestle. These homogenates were then fractionated as follows: For the low speed pellet (P1), they were centrifuged at $1000g_{av}$ for 10 min. The supernatant from this step was then centrifuged at $100,000g_{av}$ for 1 hour to yield a high speed pellet (P2; particulate) fraction, and a supernatant (SN; cytosol) fraction. The pellets were then resuspended in 0.5 ml of lysis buffer.

SDS polyacrylamide gel electrophoresis and immunoblotting- These operations were performed as described in E. Harlow & D.P. Lane, Antibodies: A Laboratory Manual 474-510 (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1990). In brief, samples were resuspended in Laemmli buffer, U.K. Laemmli, 227 Nature 680-85 (1970), and boiled for 5 min. Membranes were blocked in 5 % (w/v) low-fat milk powder in TBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) overnight at room temperature. They were then incubated with anti-PDE4D monoclonal antibody or RACK1-specific antibody diluted 1:5000 (v/v) in 1 % (v/v) low-fat milk powder in TTBS (TBS plus 0.1 % (v/v) Tween 20) for 3 hours at room temperature. Detection of the bound antibody was with anti-mouse IgG peroxidase (for PDE4D5) or anti-mouse IgM peroxidase (for RACK1) secondary antibodies (both from Sigma Chemical Co., St. Louis, Missouri) and the enhanced chemiluminescence (ECL) system (Amersham, Piscataway, New Jersey).

PDE assays- PDE activity was assayed as described in R.J. Marchmont & M.D. Houslay, 187 Biochem. J. 381-392 (1980). All assays were conducted at 30°C and in all experiments a freshly prepared slurry of Dowex:H_2O:ethanol (1:1:1; v/v) was used. In all experiments, initial rates were taken from linear time-courses of activity. K_m values were determined over a substrate range of 0.25-25 μM cAMP (7 different concentrations). Dose-dependent inhibition by rolipram was

determined in the presence of 1 μM cAMP and over a range (8-10 different values) of 10 nM to 100 mM rolipram. The IC_{50} was then determined from these values, using a least-squares fitting algorithm. Rolipram was dissolved in 100% DMSO as a 1 mM stock and diluted in 20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$ to provide a range of concentrations in the assay. The residual levels of DMSO were shown not to affect PDE activity over the ranges used in this study. To define K_m values, data from PDE assays were analyzed by computer fitting to the hyperbolic form of the Michaelis-Menten equation using an iterative least squares procedure (Ultrafit; with Marquardt algorithm, robust fit, experimental errors supplied; Biosoft, Ferguson, Missouri). Relative V_{max} values could be calculated using the Michaelis equation and the experimentally derived K_m values, as described previously. Y. Shakur et al., 306 Biochem. J. 801-809 (1995).

Measurement of protein concentrations- Protein concentrations were measured by the method of Bradford, using BSA as a standard. M.M. Bradford, 72 Anal. Biochem. 248-254 (1976).

RESULTS

Isolation of RACK1 as a protein interacting with PDE4D5 in a two-hybrid screen- PDE4D5 differs from other PDE4D isoforms by the presence of a unique amino-terminal end, 88 amino acids in length, G.B. Bolger et al., 328 Biochem J. 539-548 (1997), which has no detectable sequence homology to any other phosphodiesterase (FIG. 1).

To determine more about the properties of PDE4D5, it was determined to investigate whether specific proteins might bind to it. For this purpose, full-length PDE4D5 was used as a "bait" in a two-hybrid screen. Two independent screens were performed, with identical results. The results of one screen are shown (Table 1). In both screens, a large number of cDNA clones were obtained, all of which encoded the full ORF of the RACK1 protein. D. Ron et al., 91 Proc. Nat'l Acad. Sci. U.S.A. 839-843 (1994); D. Ron & D. Mochly Rosen, 92 Proc.

Nat'l Acad. Sci. U.S.A. 492-496 (1995); D. Ron et al., 270 J. Biol. Chem. 24180-24187 (1995); D. Mochly Rosen, 268 Science 247-251 (1995); F. Guillemot et al., 86 Proc. Nat'l Acad. Sci. U.S.A. 4594-4598 (1989).

Table 1

No. of transformants screened	24,000,000
No. of His^+ colonies isolated	3,000
No. of $His^+/LacZ^+$ colonies (of total tested)	96/96
No. of plasmids analyzed	26
No. of different clones present	2
No. of different mRNAs	1

Specificity of the PDE4D5:RACK1 interaction- To obtain preliminary evidence that the PDE4D5:RACK1 interaction was specific, two-hybrid β-galactosidase assays were used to test the interaction of RACK1 with a variety of "baits" expressed as LexA fusions. These included lamin, A.B. Vojtek et al., 74 Cell 205-214 (1993), casein kinase II, Ras, Raf, several transcription factors, and LexA itself (i.e., not as a fusion). In a similar manner, PDE4D5 was tested for its ability to bind to these proteins expressed as GAL4 fusions, and also to the GAL4 activation domain itself (i.e., not as a fusion). No interaction was detected under conditions where an interaction could be shown between PDE4D5 and RACK1 (data not shown). PDE4D5 was also tested for its ability to bind to two other WD-repeat proteins, the G-protein $G_s\beta$ subunit and the coatomer subunit protein, β'-COP. The rationale for testing β'-COP was that, like RACK1, it has also been shown to bind to PKC isoforms, although with selectivity for different PKC isoforms than RACK1. M. Csukai et al., 272 J. Biol. Chem. 29200-29206 (1997). An interaction between PDE4D5 and either of these two WD-repeat proteins was not detected using β-galactosidase assay conditions that did detect the interaction between PDE4D5 and RACK1 (The results for β'-COP are shown in

FIG. 2). These data suggest that PDE4D5 interacts specifically with RACK1 and not with WD-repeat proteins generally.

Endogenous expression of RACK1 and PDE4D5 in COS7 cells- To confirm and expand these two-hybrid data, it was tested whether PDE4D5 and RACK1 could be co-immunoprecipitated from mammalian cells. Experiments were performed first on COS7 cells (this and the next two sections) and then were later expanded to the study of other cell types (see below).

It has been previously determined that PDE4D exists both in the cytosol and in the cellular particulate fraction of various tissues and cell lines. G.B. Bolger et al., 328 Biochem J. 539-548 (1997). To determine whether this was also true in COS7 cells, COS7 cells were disrupted and fractionated to yield a high speed (S2) supernatant fraction reflecting cytosolic components, and also P1 and P2 particulate fractions (see Experimental Procedures). The fractions were subjected to SDS-PAGE and immunoblotted with antibodies specific for either PDE4D or RACK1. Both specific polyclonal and monoclonal PDE4D antibodies were used, with similar results. G.B. Bolger et al., 328 Biochem J. 539-548 (1997). The PDE4D antibodies were all generated to a carboxy-terminal region of the PDE4D protein and have been shown to detect all five PDE4D species, G.B. Bolger et al., 328 Biochem J. 539-548 (1997), as the PDE4D isoforms differ solely by their distinct amino-terminal regions (FIG. 1).

Analysis of untransfected COS7 cells showed that they contain two PDE4D immunoreactive species of 95±3 and 103±2 kDa (FIG. 3A), consistent with the endogenous expression of the PDE4D3 and PDE4D5 isoforms, respectively. G.B. Bolger et al., 328 Biochem J. 539-548 (1997). Recombinant PDE4D3 and PDE4D5 isoforms expressed transiently in COS7 cells, G.B. Bolger et al., 328 Biochem J. 539-548 (1997), co-migrated on SDS-PAGE with these two endogenous PDE4D species (data not shown). As demonstrated previously, G.B. Bolger et al., 328 Biochem J. 539-548 (1997), when COS7 cells were transfected with cDNAs encoding these two isoforms, so that the recombinant enzymes represented greater than 98% of total PDE activity in these

cells, the major fraction of these enzymes was located in the cytosol (S2) fraction, but some immunoreactivity was also seen in the pellet fractions.

The specific antibody to RACK1 detected in untransfected COS7 cells a single 36±1 kDa species (FIG. 3B), indicative of the presence of endogenous RACK1. D. Ron et al., 91 Proc. Nat'l Acad. Sci. U.S.A. 839-843 (1994). As also found for PDE4D5, the major fraction of RACK1 was located in the cytosolic (S2) fraction, although RACK1 immunoreactivity was also evident in the pellet fractions, to a similar extent to the level seen for endogenous PDE4D5 (FIG. 3A).

PDE4D5 and RACK1 can be co-immunoprecipitated from COS7 cells- To determine whether endogenous PDE4D5 and RACK1 interacted in COS7 cells, the cytosolic (S2) fraction from COS7 cells was subjected to an immunoprecipitation protocol using either the RACK1 antibody (FIG. 3C; lanes marked "RACK1") or the PDE4D antibody (FIG. 3C; lanes marked "4D"). The resulting immunoprecipitates were then subjected to SDS-PAGE and immunoblotting. Since PDE4D5 migrates as a 105 kDa protein, G.B. Bolger et al., 328 Biochem J. 539-548 (1997), and RACK1 as a 36 kDa protein, D. Ron et al., 91 Proc. Nat'l Acad. Sci. U.S.A. 839-843 (1994), the top half of the blot was treated with the PDE4D-specific antibody, and the bottom half with the RACK1-specific antibody. Cytosolic extracts (i.e., not subjected to immunoprecipitation) were immunoblotted as controls (FIG 3C; lanes marked "none"). From this analysis, it was clear that the RACK1 antibody not only immunoprecipitated RACK1 but that it also co-immunoprecipitated PDE4D5 (FIG. 3C). Conversely, the PDE4D antibody not only immunoprecipitated PDE4D3 and PDE4D5 but it also co-immunoprecipitated RACK1. These data demonstrate that endogenously expressed PDE4D5 and RACK1 are in a complex in the cytosol of COS7 cells. It was also shown that particulate RACK1 and PDE4D5, when solubilized by an NP-40 detergent system described in B.Y. Chang et al., 18 Mol. Cell. Biol. 3245-3256 (1998), could be similarly co-immunoprecipitated (data not shown).

PMA does not affect binding of RACK1 to PDE4D5- It has

been demonstrated previously that treatment of cells with phorbol esters, which activate PKC, is a necessary prerequisite for the interaction of RACK1 with both PKC, D. Ron et al., 91 Proc. Nat'l Acad. Sci. U.S.A. 839-843 (1994), and the integrin β-subunit, J. Liliental & D.D. Chang, 273 J. Biol. Chem.. 2379-2383 (1998), but not for the interaction of RACK1 with Src, B.Y. Chang et al., 18 Mol. Cell. Biol. 3245-3256 (1998). The present co-immunoprecipitation data suggest that PDE4D5, like Src, can interact with RACK1 without PKC activation, since the treatment of COS7 with the phorbol ester, PMA, did not affect the ability of PDE4D5 and RACK1 to be co-immunoprecipitated, regardless of which antibody was used (FIG. 3C). To ascertain that PMA was able to activate PKC in COS7 cells, COS7 cells were treated with PMA and harvested at various times, and cytosolic (S2) fractions were prepared therefrom and subjected to SDS-PAGE and immunoblotting with antibodies for PKCα (FIG. 3D), RACK1 (FIG. 3E), or PDE4D (FIG. 3F). These experiments showed clearly that endogenous PKCα was rapidly and completely translocated from the cytosol to the particulate fraction within 5-10 min (FIG 3D). These data also indicate that PKCα was not constitutively activated in COS7 cells, as PKCα was clearly cytosolic prior to challenge with PMA. These observations suggest that PDE4D5, like Src, can interact with RACK1 without PKC activation. Additionally, they suggest that PMA does not trigger any translocation of either RACK1 or PDE4D5 to the particulate fraction of COS7 cells (FIGS. 3E and 3F).

PDE4D5 and RACK1 can be co-immunoprecipitated from various cell types- To determine whether the PDE4D5:RACK1 interaction occurred in cells generally, a number of other cell lines were examined, specifically HEK293 (human embryonic kidney), 3T3-F442A (mouse fibroblast/pre-adipocyte), SK-N-SH (human neuroblastoma), and Jurkat (human T-cell). Cytosolic extracts from these cell lines were subjected to SDS-PAGE with subsequent immunoblotting. The top half of the immunoblot was treated with a PDE4D antibody and the bottom half with the RACK1 antibody (FIGS. 4A and 4B, lanes marked "T"). With the PDE4D antibody, two immunoreactive species of 105±3 kDa and

95±2 kDa were detected in extracts from both HEK293 and 3T3-F442A cells (FIGS. 4A and 4B, lanes marked "T"). These isoforms co-migrated (data not shown) with recombinant PDE4D5 (105 kDa) and PDE4D3 (95 kDa; G.B. Bolger et al., 328 Biochem J. 539-548 (1997)), respectively, and migrated very distinctly from the other PDE4D isoforms, G.B. Bolger et al., 328 Biochem J. 539-548 (1997), namely PDE4D1 (68 kDa), PDE4D2 (68 kDa) and PDE4D4 (119 kDa). A faint band indicative of an ~68 kDa immunoreactive species was observed in immunoblots of 3T3-F442A cells (FIG. 4B), which may reflect either or both of the PDE4D1/2 isoforms. G.B. Bolger et al., 328 Biochem J. 539-548 (1997). In contrast, a single ~105 kDa immunoreactive species, indicative of PDE4D5, G.B. Bolger et al., 328 Biochem J. 539-548 (1997), was noted in SK-N-SH cells (FIG. 4B) and no immunoreactive PDE4D species was noted in cytosolic extracts from Jurkat cells (FIG. 4A). Immunoblotting of the lower half of the gel with an antibody specific for RACK1 identified a single 36±2 kDa species present in all of these cell types (FIGS. 4A and 4B, lanes marked "T").

To test for an interaction between PDE4D5 and RACK1 in cytosolic extracts from these cell lines, they were subjected to an immunoprecipitation protocol with either the RACK1 antibody (FIGS. 4A and 4B; lanes marked "i" or "rIP") or a mouse non-specific antiserum (FIGS. 4A and 4B; lanes marked "n"). The resulting immunoprecipitates were then subjected to immunoblotting with the PDE4D and RACK1 antibodies, as described above. These experiments showed that RACK1 could be immunoprecipitated from all cells by the RACK1 antibody, but not by the non-specific antiserum. In the HEK293, 3T3-F442A, and SK-N-SH cell lines, all of which express the PDE4D5 isoform, a single 105 kDa species in the RACK1 immunoprecipitates was detected upon immunoblotting with a PDE4D antibody (FIG. 4B). No PDE4D immunoreactive species was detected in RACK1 immunoprecipitates from Jurkat T-cells, which did not express PDE4D5 (FIG. 4A). These data indicate that natively expressed cytosolic RACK1 is complexed with natively expressed cytosolic PDE4D5 in both human (HEK293 and SK-N-SH) and mouse (3T3-F442A) cell lines. These data are

consistent with PDE4D5 being quantitatively immunoprecipitated with RACK1 from HEK293 and 3T3-F442A cells. Additionally, the data support the concept that PDE4D3 does not interact with RACK1, since PDE4D3 was not immunoprecipitated from HEK293 and 3T3-F442A cells using the RACK1 antibody (FIG. 4B).

PDE4D5 and RACK1 interact directly in vitro *in a dose-dependent manner*- To determine whether RACK1 and PDE4D5 interacted directly, rather than through an intermediate protein, the interaction between recombinant RACK1 and PDE4D5 as synthesized in *E. coli* was studied. PDE4D5 was expressed as a maltose-binding protein fusion (MBP-PDE4D5) in *E. coli*. It was then purified (FIG. 5B) on a maltose affinity column (see Experimental Procedures) and had an activity of 29 ± 2 pmol/min/mg protein (with 1 μM cAMP as substrate) and a K_m value of 5.1 ± 0.7 μM cAMP (mean ± standard deviation; n=3 experiments). RACK1 was generated and purified as a GST fusion in *E. coli* (GST-RACK1; FIG. 5A; see Experimental Procedures).

To measure the interaction of PDE4D5 with RACK1, the purified protein was used in a capture plate assay. This demonstrated (FIG. 5C) that PDE4D5 bound to RACK1 in an apparent dose-dependent fashion. From these experiments the affinity of the interaction between RACK1 and PDE4D5 was determined as having an EC_{50} of 7.4 ± 1.1 pM (mean±SD; n=3 experiments). These data demonstrate that RACK1 and PDE4D5 can interact directly without any intermediary protein being involved, and that the interaction is unlikely to require any post-translational modification of the two proteins. The high affinity for interaction is also compatible with a direct interaction *in vivo*.

It was also demonstrated that PDE4D3, as a purified MBP fusion protein (FIG. 5B), was unable to bind to RACK1 when the capture assay was performed under conditions identical to those that demonstrated interaction between RACK1 and PDE4D5 (FIG. 5C). This is consistent with the lack of interaction between PDE4D3 and RACK1 in intact cells (FIGS. 3C and 4B).

Enzymatic activity of RACK1-bound PDE4D5- To evaluate

whether interaction with RACK1 altered the catalytic activity of PDE4D5, the effect on PDE4D5 activity of complexing *E. coli*-purified recombinant PDE4D5 with *E. coli*-purified recombinant RACK1 was tested. Under conditions (using a "pull-down" procedure; see Experimental Procedures) where all of the PDE4D5 could be shown to be complexed with RACK1, it was found that RACK1 produced little or no change in either the K_m or V_{max} for the hydrolysis of cAMP by PDE4D5. Specifically, PDE4D5 exhibited a K_m of 5.1 ± 0.7 μM cAMP when free from RACK1 and a K_m of 6.9 ± 0.9 when complexed with RACK1 (mean ± SD; n=3 experiments). The ratio of maximal catalytic activity for cAMP hydrolysis between RACK1-bound and free forms of PDE4D5 was 0.95 ± 0.06 (mean ± SD; n=3 experiments). The effect of RACK1 on the thermal denaturation profile of PDE4D5, which was measured upon incubation of the enzyme at 50° C, was also determined. Whether complexed or not to RACK1, the activity of PDE4D5 decayed as a single exponential (data not shown), indicative of a single homogenous enzyme in both instances. The half-lives for decay of PDE activity were very similar for both the RACK1-complexed and uncomplexed forms of PDE4D5 (38.3 ± 2.1 and 34.3 ± 2.6 min; mean ± SD; n=3 experiments). This suggests that interaction of PDE4D5 with RACK1 does not elicit a major conformational change affecting enzyme activity.

A number of investigators have noted that PDE4 isoforms can undergo conformational changes, which alter their sensitivity to inhibition by rolipram and other PDE4-selective inhibitors. E. Huston et al., 271 J. Biol. Chem. 31334-31344 (1996); R. Alvarez et al., 48 Mol. Pharmacol. 616-622 (1995); J.E. Souness & S. Rao, 9 Cell Signal. 227-236 (1997). Therefore, the sensitivity of *E. coli*-purified MBP-PDE4D5 to rolipram was analyzed when both free and complexed with *E. coli*-purified GST-RACK1. Dose-effect analyses (FIG. 5D) showed that rolipram inhibited the cAMP PDE activity of MBP-PDE4D5 in a dose-dependent manner, with an IC_{50} value of 0.16 ± 0.5 μM. In contrast, when this enzyme was complexed with GST-RACK1 a small shift in sensitivity to rolipram inhibition was

seen (FIG. 5D), which was reflected in an increase in the IC_{50} to 0.52±0.07 μM. This effect was unlikely to be caused by the GST portion of GST-RACK1, as the addition of GST alone to the assays, at a level identical to that of GST-RACK1, had negligible effect on rolipram inhibition (FIG. 5D; IC_{50} of 0.19 ± 0.02 μM).

RACK1 interacts with PDE4D5, but not with other PDE4 isoforms- Five PDE4D isoforms have been identified to date (G.B. Bolger et al., 328 Biochem J. 539-548 (1997); FIG. 1). It has already been demonstrated herein that RACK1 does not interact with PDE4D3 (FIGS. 3C, 4B, 5C). To determine whether any of the PDE4D1, PDE4D2, PDE4D3, or PDE4D4 isoforms interact with RACK1, cDNAs encoding these isoforms were expressed in *S. cerevisiae* as LexA fusions and two-hybrid assays were used to test for their ability to interact with a GAL4-RACK1 fusion. No interactions were detected (data not shown), indicating that RACK1 interacts specifically with the PDE4D5 isoform.

To confirm this observation in mammalian cells, the ability of RACK1 to associate with recombinant PDE4 isoforms expressed in COS7 cells was also tested. For these experiments, a GST "pull-down" method, as modified previously, J.C. O'Connell et al., 318 Biochem. J. 255-262 (1996), was used. In brief, COS7 cells were transiently transfected with cDNAs encoding various PDE4 isoforms. Cytosolic fractions were prepared from the transfected cells and incubated with GST-RACK1, and the resulting complex absorbed onto glutathione agarose beads (see Experimental Procedures). The beads were then harvested by centrifugation, washed, and immunoblotted with the PDE4D antibody (FIGS. 6A-C).

As shown before, G.B. Bolger et al., 328 Biochem J. 539-548 (1997); I. McPhee et al., 310 Biochem. J. 965-974 (1995); Y. Shakur et al., 306 Biochem. J. 801-809 (1995); G.B. Bolger et al., 271 J. Biol. Chem. 1065-1071 (1996); E. Huston et al., 271 J. Biol. Chem. 31334-31344 (1996); E. Huston et al., 328 Biochem J. 549-556 (1997), in homogenates of the transfected COS7 cells the activity of the transfected PDE4D species accounted for over 98% of the total cellular cAMP PDE activity

(FIG. 6A, lanes marked "ly"). When cytosolic extracts from PDE4D5-transfected cells were subjected to "pull-downs" with GST-RACK1, a 105 kDa PDE4D immunoreactive species consistent with PDE4D5 was identified on the immunoblots (FIG. 6A, lanes marked "rg"). In contrast, when pull-downs were performed with GST alone, no PDE4D species was seen on the immunoblots (FIG. 6A, lanes marked "g"). In parallel experiments, pull-downs were performed from COS7 cells transfected with a cDNA encoding PDE4D3, however, no immunoreactive PDE4D3 was pulled down with GST-RACK1 (FIG. 6A). However, endogenous PDE4D5 was pulled down in these experiments to a level similar to that seen using an equivalent amount of cytosol from mock transfected cells.

In similar experiments, it was determined that PDE4D1, PDE4D2 and PDE4D4, when expressed in COS cells, G.B. Bolger et al., 328 Biochem J. 539-548 (1997), could not be pulled down by GST-RACK1 (data not shown). In still additional experiments, it was determined that the human PDE4A4/5 isoform, G. Bolger et al., 13 Mol. Cell Biol. 6558-6571 (1993); E. Huston et al., 271 J. Biol. Chem. 31334-31344 (1996), the human PDE4B isoforms PDE4B1, PDE4B2, and PDE4B3, E. Huston et al., 328 Biochem J. 549-556 (1997), and also the human PDE4C2 isoform, R.K. Sunahara et al.,36 Annu. Rev. Pharmacol. Toxicol. 461-480 (1996), when expressed in COS7 cells, could not be pulled down with GST-RACK1. These data imply that, of the known PDE4 isoforms, RACK1 binds selectively to PDE4D5.

The interaction of PDE4D5 with RACK1 is mediated by specific amino acids in the PDE4D5 amino-terminal region- Since RACK1 interacts specifically with PDE4D5, and not with other PDE4 isoforms, it is likely that RACK1 interacts with regions of sequence that are unique to PDE4D5. PDE4D5 differs from all known PDE4 isoforms, M.D. Houslay et al., 44 Advances in Pharmacology 225-342 (1998); G.B. Bolger et al., 328 Biochem J. 539-548 (1997), in the presence of a unique region of 88 amino acids at its amino-terminus (FIG. 1; SEQ ID NO:1). It was decided to determine which specific amino acids within this region are essential for the binding of PDE4D5 to RACK1.

As a first step, a two-hybrid construct containing just the unique 88 amino acid amino-terminal region of PDE4D5 was created, and it was demonstrated that it could interact with RACK1 (FIG. 7A, patches marked "NT"). Constructs encoding deletion mutations in the amino-terminal region of PDE4D5 were then constructed and tested for their ability to interact with RACK1. The interaction was tested by both a two-hybrid assay (FIG. 7A) and by pull-down experiments (FIG. 6B). For the two-hybrid experiments, constructs encoding fusions between LexA and various amino-terminal deletions of PDE4D5 were tested for interaction with GAL4-RACK1. For the pull-down experiments, the ability of mutant PDE4D5 forms expressed in COS7 cells to interact with GST-RACK1 were tested. For the COS7 cell experiments, it was essential that the transfected mutant protein and endogenous PDE4D5 proteins in COS7 cells could be distinguished. For this purpose, PDE4D5 constructs that encoded an epitope of the vesicular stomatitis virus envelope, T.E. Kreis, 5 EMBO J. 931-941 (1986), attached to the carboxyl-terminal end of the protein, G.B. Bolger et al., 328 Biochem J. 539-548 (1997); R. Hoffmann et al., 333 Biochem J 139-149 (1998), were used. Expression of these VSV-tagged PDE4D5 species in COS7 cells could be detected by immunoblotting with a monoclonal antibody specific for the VSV epitope. T.E. Kreis, 5 EMBO J. 931-941 (1986). Preliminary experiments demonstrated that COS7-cell expressed, VSV-tagged unmutated PDE4D5 could be pulled down with GST-RACK1 (FIG. 6B, lane "wt"). In contrast, COS7 cells transfected with the vector alone demonstrated no detectable VSV immunoreactivity (FIG. 6B, lane "m"). The ability of the various PDE4D5 amino-terminal deletion mutants to interact with GST-RACK1 was then tested. The pull-down experiments (FIG. 6B) demonstrate that a single region of the PDE4D5 amino terminus, comprising amino acids 12 through 29, is necessary for interaction with RACK1, but that other regions appear to be dispensable. Identical conclusions were obtained using two-hybrid methods (FIG. 7A).

To obtain additional data localizing the region of interaction, two mutants with deletions in the middle of the PDE4D5 amino terminal region were created. One deletion (D2,

SEQ ID NO:17, FIG. 1) removed amino acids 22 to 27 and the other (D1, SEQ ID NO:16) amino acids 19 through 51. Neither of these two mutations interacted with GST-RACK1 in a pull-down assay (FIG. 6B). These data narrowed down a region essential for interaction with RACK1 to a small region within the PDE4D5 amino-terminus. Secondary structure analyses performed with the Wisconsin package of DNA analysis programs (data not shown) demonstrated that this region of PDE4D5 (approximately amino acids 19-50) may form an α-helix, but otherwise did not have any distinguishing sequence motifs. It has no obvious primary sequence homology to PKC, Src, or integrins.

The effects of mutations in individual amino acids in the region defined by the deletion mutation analysis were then analyzed. Site-directed mutagenesis was used to mutate individual codons in this region to alanine. The effect of each of these mutations on the interaction with RACK1 was tested with the two-hybrid assay. Ten codons were individually mutated (SEQ ID NO:2 through SEQ ID NO:11), and mutations of four of these (Asn22, SEQ ID NO:6; Pro23, SEQ ID NO:7; Trp24, SEQ ID NO:8;and Asn26, SEQ ID NO:10) were each shown to completely block the interaction (FIG. 7B). Two of these mutations (Asn22 and Trp24) were then expressed in COS7 cells and tested for their ability to co-immunoprecipitate with RACK1. Both the mutations blocked the interaction in this assay as well (FIG. 6C). These mutations were clustered in a very small region of the protein, which presumably serves as the major, or possibly only, region of interaction between PDE4D5 and RACK1.

Example 1

Using procedures described in G. Scotland & M.D. Houslay, 308 Biochem. J. 673-681 (1995), and K.J. Smith et al., 271 J. Biol. Chem. 16703-16711 (1996), PDE4D5 was synthesized *in vitro* using the coupled, single tube STP3T7 transcription/translation system (Novagen, Inc., Madison, Wisconsin) according to the manufacturer's instructions. Briefly, 1μg of pcDNA4D5VSV DNA template was added to STP3

Transcription Mix (10 μl total reaction volume) and incubated at 30°C for 15 minutes. Following the transcription step, 30 μl of STP3 Translation Mix and 30 μCi of [^{S}35]-methionine were added to the reaction tube. The reaction volume was made to 50 μl with nuclease-free water, and then the tube was incubated at 30°C for 60 minutes. This product was then used in binding experiments with GST-RACK1.

For binding experiments, 5 μg GST-RACK1/reaction was incubated in the presence or absence of 100 μM of the competition peptides f39 (SEQ ID NO:18), which corresponds to the RACK1 binding region of PDE4D5, or 1-25 RD1 (SEQ ID NO:19), which corresponds to the N-terminal region of PDE4A1, in a total volume of 200 μl binding buffer (55 mM Tris-HCl, pH7.4, 132 mM NaCl, 22 mM sodium fluoride, 11 mM sodium pyrophosphate, 1.1 mM EDTA, 5.5 mM EGTA, and protease inhibitor mixture), for 1 hour at 4°C. Following this, 5 μl of *in vitro*-synthesized product prepared according to the procedure described above and 30 μl (packed volume) of glutathione Sepharose beads were added and the incubation continued for a further 3 hours. The beads were collected by centrifugation (2,000xg_{av}, 5 minutes, 4°C), washed three times with binding buffer and subjected to SDS-PAGE. Radioactive bands were identified by PhosphoImager analysis.

Using the procedures outlined above, the f39 peptide (SEQ ID NO:18) completely blocked the interaction between RACK1 and PDE4D5.

Example 2

Two phage libraries used for library screening were the M13-phage-based heptapeptide (Ph.D.-7) and dodecapeptide (Ph.D.-12) random display libraries supplied by New England Biolabs, Inc. (Beverly, Massachusetts). For library screening GST-RACK1 or GST-LYN fusion proteins or GST protein were used. Proteins were immobilized on pre-blocked, glutathione coated ELISA plates (Pierce, Rockford, Illinois) by direct coating with 100 μl of a 10 mg/ml (w/v) solution of the protein in TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 16 hours at 4°C in a

humidified container. The coating solution was discarded and plates were washed rapidly 6 times with TBST (TBS + 0.1% (v/v) Tween-20). Before each library was screened against fusion proteins, three rounds of pre-clearing with GST were carried out to remove non-specific phage particles from libraries. Approximately 1 x 10^{12} phage particles in TBS (100 μl total volume) were added sequentially to three GST-coated wells.

Pre-binding reactions were incubated for 10 minutes at room temperature. Pre-cleared phage were then added to a well coated with either GST, GST-RACK1, or GST-LYN and incubated for 1 hour at room temperature. Unbound phage were removed by washing the wells 10 times with cold TBST. Bound phage were eluted from wells by incubating for 2 minutes with 100 μl of 5 mM glutathione, 50 mM Tris, pH 8.0. The elution step was repeated two additional times and then the eluates were pooled. Eluted phage were amplified by infecting mid-log phase (OD_{600}=0.5) *E. coli* (strain ER2537) in 20 ml of LB media (10 g/l (w/v) tryptone, 5 g/l (w/v) yeast extract, 5 g/l (w/v) NaCl) and incubating with vigorous shaking at 37°C for 4.5 hours. Phage were harvested by removing the bacteria by centrifugation (10,000xg_{max}, 10 minutes, room temperature) and precipitating the phage-containing supernatant overnight at 4°C with 1/6 volume polyethylene glycol (PEG)/NaCl (20% (w/v) PEG-8000, 2.5 M NaCl). The PEG precipitate was collected by centrifugation (10,000xg_{max}, 10 minutes, 4°C) and resuspended in 1 ml TBS. Residual bacterial cells were removed by centrifugation at 13,000xg_{max} for 5 minutes at 4°C and then the supernatant re-precipitated for 60 minutes at 4°C with 1/6 volume PEG/NaCl. The amplified eluate was collected by centrifugation (13,000xg_{max}, 10 minutes, 4°C) and resuspended in 200 μl TBS, 0.02% NaN_3. The amplified phage pool was then pre-cleared, added to GST or GST-fusion protein coated wells, incubated, eluted and amplified as described above except that wash steps carried out with TBS + 0.5% (v/v) Tween 20. Four cycles of affinity enrichment were carried out for the Ph.D.-12 library and three rounds of enrichment for the Ph.D.-7 library.

Phage titers (of primary and amplified libraries, eluted

and amplified phage) were determined following infection of 200 μl of mid-log phase *E. coli* ER2537 with 10 μl of LB-diluted phage for 5 minutes at room temperature. Infected cells were then transferred to a culture tube containing 3 ml of pre-warmed (45°C) Agarose Top (10 g/l (w/v) tryptone, 5 g/l (w/v) yeast extract, 5 g/l (w/v) NaCl, 1 g/l $MgCl_2.6H_20$, 7 g/l agarose) and immediately poured onto pre-warmed (37°C) LB/isopropyl β-D-thiogalactoside (IPTG)/5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal) plates (LB medium containing 15 mg/ml (w/v) agar, 80 μg/ml (w/v) IPTG, 3 μg/ml (w/v) Xgal). Plates were incubated overnight at 37°C and the number of resulting blue colonies was multiplied by the phage dilution factor for that plate to give the titer in plaque forming units (pfu) per 10 μl. To amplify individual phage clones for ELISA binding analysis and DNA sequencing, single colonies were isolated from the titering plates and used to infect 1 ml of a 1:100 (v/v) dilution of an overnight culture of ER2537 *E. coli* . Cultures were incubated for 5 hours with shaking at 37°C and the phage-containing supernatant was cleared of bacteria by centrifugation (13,000xg_{max}, 30 seconds, 4°C). For DNA sequencing 500 ml of the phage stock was precipitated with 200 μl PEG/NaCl and the phage-containing pellet resuspended in 100 μl iodide buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 4 M NaI). Preferential precipitation of single-stranded phage DNA was accomplished by the addition of 250 μl of ethanol followed by incubation for 10 minutes at room temperature. Precipitated DNA was collected by centrifugation (13,000xg_{max}, 10 minutes, 4°C), washed with 70% (v/v) ethanol/H_2O and dried under vacuum. The DNA pellet was then resuspended in 30 μl of TE buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA). DNA sequencing of recombinant phage inserts was done by the dideoxy chain termination technique using the fluorescent dye mixture for automated sequencing (ABI) and the -96gIII sequencing primer supplied by New England Biolabs.

To assess RACK1 binding properties of individual phage clones and selected peptides an ELISA system was used. The peptides used were f39 (SEQ ID NO:18), which corresponds to

the RACK1 binding region of PDE4D5, and a 25mer peptide (SEQ ID NO:19) that corresponds to the N-terminal region of PDE4A1. ELISA plate wells were coated with peptide (10 μg) or selected phage clones (1 x 10^{12} virions) diluted in 200 μl of 0.1 M $NaHCO_3$ (pH 8.6) and incubated overnight at 4°C in an air-tight humidified container. Plates were then blocked for 2 hours at 4°C with 200 μl/well of 5 mg/ml BSA in 0.1 M $NaHCO_3$ (pH 8.6). Wells were washed 6 times with TBST (TBS + 0.5% (v/v) Tween-20) and then incubated for 1 hour at room temperature with GST-RACK1 (in a range of concentrations of 2 mM to 50 pM) diluted in TBST) and then the wells were washed 6 times with ice cold TBST. The primary antibody, monoclonal anti-RACK1 antiserum (Transduction Laboratories, Lexington, Kentucky) diluted 1:5000, was added and incubated for 1 hour at room temperature. Wells were then washed 6 times with ice cold TBST. The secondary antibody, horse radish peroxidase (HRP) conjugated goat anti-mouse IgM (Sigma), was added and incubated for 1 hour at room temperature. Wells were then washed as before. Color formation was initiated with the addition of the HRP substrate 0.022% (w/v) 2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma) in 50 mM sodium citrate, pH 4.0, containing 5 x 10^{-5}% H_2O_2. Plates were read using a microplate reader set at 405 nm.

For library screening, two M13 phage-based pIII-protein display libraries were screened against GST, GST-RACK1 and GST-LYN-SH3. One of these consisted of 7 random residues linked to the pVIII protein by a linker of three tandem copies of the sequence Gly_4Ser. For the 7-mer library a total of three rounds of enrichment cycles, affinity selection and amplification were performed, whereas four rounds were performed for the 12-mer library. To enhance the possibility of isolating RACK1 and LYN-SH3, domain-specific phage libraries were incubated with immobilized GST or MBP prior to affinity selection. A phage-based ELISA was employed to assess the binding specificity of individual phage clones.

For each of the phage libraries screened with RACK1 and LYN-SH3 phage, clones were examined after each of the third and fourth rounds of screening. The deduced amino acid

sequences of the recombinant peptide extension of GST, GST-RACK1, and GST-LYN-SH3 phage are shown in Tables 2-7. The 7-mer library showed rapid enrichment kinetics during the rounds of affinity selection when screened against LYN-SH3 and GST, displaying recognizable sequence characteristics after three rounds of enrichment. In agreement with R.J. Rickles et al., 92 Proc. Nat'l Acad. Sci. USA 10909-10913 (1995), the highest frequency of LYN-SH3-specific phage recovered contained the class I ligand consensus motif Arg-Pro-Leu-Pro-Pro-Leu-Pro (SEQ ID NO:26). In contrast, the majority of GST-specific phage contained the sequence motif His-His-Ser-His-Thr Pro Arg (SEQ ID NO:21) demonstrating the specificity of the screening process for the LYN-SH3 fusion-partner protein and not for GST. RACK1 phage did not contain any of the sequence characteristics of LYN-SH3- or GST-specific clones. However, no consistent sequence motifs were obtained from RACK1 phage screened against the 7-mer library. Moreover, this suggests that the 7mer library expresses an insufficient number of residues to obtain a minimal consensus for RACK1-binding. Examination of the sequences from the third RACK1 enrichment cycle of the 12-mer library revealed that, most notably, all RACK1 phage contained the sequence motif Pro-Xaa-Leu. However, the LYN-SH3 phage from the same library and enrichment cycle had incomplete binding consensus for SH3 domains. The 12-mer library was therefore subjected to a further round of enrichment purification against LYN-SH3 and RACK1. Following this, LYN-specific phage contained recognizable SH3-domain binding motifs. All recovered RACK1-specific clones contained the sequence motif Pro-Xaa-Leu. In the greatest number of enriched RACK1 phage, Xaa is a hydrophobic amino acid (Pro or Ala). In addition to the hydrophobic Pro-Xaa-Leu core, all RACK1 phage were rich in other hydrophobic amino acids (Leu, Ile, Ala, Phe, and Trp), which were found carboxy- and amino-terminal to the Pro-Xaa-Leu motif. Other sequence characteristics of the collection of RACK1 phage included a propensity for an acidic amino acid (Asp or Glu), which only occurred amino terminal to the Pro-Xaa-Leu motif. The most abundantly enriched RACK1-specific

phage also contained a strongly basic lysine residue carboxy terminal to the Pro-Xaa-Leu motif. The organization of amino acids upstream of the proline and downstream of the lysine residues yielded the mixed consensus sequence Glu-Xaa-Pro-Xaa-Leu (SEQ ID NO:51).

Tables 2-6 show the amino acid sequences of the randomized regions of GST, LYN-SH3, and RACK1 specific phage.

Table 2. GST-specific Phage from the 7-mer Library		
Amino Acid Sequence	Frequency (%)	SEQ ID NO:
His His Thr His Thr Met Arg	30	20
His His Ser His Thr Pro Arg	20	21
His His Ser His Ala Ser Arg	10	22
His His Ser His Thr Pro Pro	20	23
Gly His His Pro Thr Ile Arg	10	24
Asn Pro His Phe His Lys Pro	10	25
His His Ser His Thr Pro Arg	Consensus	21

Table 3. LYN-SH3-specific Phage from the 7-mer Library		
Amino Acid Sequence	Frequency (%)	SEQ ID NO:
Arg Pro Leu Pro Pro Leu Pro	55.6	26
Arg Pro Leu Pro Pro Ile Pro	33.3	27
Gln Pro Thr Ser Ser Leu Pro	11.1	28
Arg Pro Leu Pro Pro Leu Pro	Consensus	26

Table 4. RACK1-specific Phage from the 7-mer Library		
Amino Acid Sequence	Frequency (%)	SEQ ID NO:
Thr His Pro Ile Asn Ser Glu	14.3	29
Lys Pro His Val Asn Val Ser	7.1	30
Ile Ser Thr Pro Gln Cys Glu	7.1	31
Lys Pro Ser Ser Met Asp Thr	7.1	32
Thr Ala Leu Pro Ser Gly Gln	7.1	33
Ser Pro Ser Pro Ile Leu Gln	7.1	34
Asn Gly Ala Ser Ser Pro Ile	7.1	35

His Met Asn Ala His Ser Pro	7.1	36
His Ser Leu Ile Leu Leu Ala	7.1	37
Gln Pro Ser Glu Leu Gln Val	7.1	38
Thr Gln Tyr Glu Ile Trp Arg	7.1	39
His Lys Glu Phe Arg Leu Leu	7.1	40
Ser Pro Tyr Ser Leu Leu Gly	7.1	41
Ser Pro Ser Pro Ile Leu Gln	Consensus	34

Table 5. LYN-SH3-specific Phage from the 12-mer Library

Amino Acid Sequence	Frequency (%)	SEQ ID NO:
Thr Arg Pro Leu Pro Pro Val Pro Thr Lys Leu His	16.7	42
Tyr His Thr Thr Pro Leu Pro Leu Pro Pro Ala Gly	16.7	43
His Tyr Pro Ser Asn Arg Pro Leu Pro His Leu Pro	16.7	44
His His Leu Ser Thr Arg Ser Leu Pro Gln Leu Pro	16.7	45
Ser Arg Pro Leu Pro Pro Leu Pro Ser Leu Leu Val	16.7	46
Glu His Arg Thr Tyr Pro Leu Pro Pro Phe Thr Leu	16.7	47
Arg Pro Leu Pro Pro Leu Pro	Consensus	26

Table 6. RACK1-specific Phage from the 12-mer Library

Amino Acid Sequence	Frequency (%)	SEQ ID NO:
Ser His Trp Glu Trp Pro Pro Leu Lys Val Pro Ser	63.6	48
Ala Leu Ile Leu Asp Thr Thr Glu Trp Pro Ala Leu	18.2	49
Ser Ile Trp Tyr Asp Leu Asn Phe Pro Thr Leu Gly	18.2	50
Ser His Trp Glu Trp Pro Pro Leu Lys Val Pro Ser	Consensus	48

The peptides identified in this screen are as follows: (1) Ser-His-Trp-Glu-Trp-Pro-Pro-Leu-Lys-Val-Pro-Ser (SEQ ID NO:48) from the 12mer library, and (2) Ser-Pro-Ser-Pro-Ile-Leu-Gln (SEQ ID NO:34) from the 7-mer library. Note that both of these peptides contain the Pro-Xaa-Leu motif present in the amino-terminus of PDE4D5.